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THE EFFECT OF HYPERVITAMINOSIS A ON EMBRYONIC LIMB-BONES CULTIVATED *IN VITRO*

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Experiments on young animals have shown (Mellanby, 1938, 1939, 1944, 1947) that vitamin A determines the shape and texture of certain bones by controlling the position of the osteoblasts and osteoclasts of periosteal bone and the intensity of their activity. Both deficiency and excess of the vitamin cause severe changes in the skeleton, though the way in which the vitamin acts remains obscure.

Some years ago a method was developed for cultivating early bone rudiments *in vitro* (Fell & Robison, 1929; Fell, 1951); the explants grew and continued to differentiate *in vitro* in a surprisingly normal way. It occurred to Mellanby (1944) that such bone cultures might be used to study the action of vitamin A on skeletal tissue and especially to decide whether the action was a direct one. The method of cultivating organized tissues *in vitro* may be a valuable adjunct to animal experiments because of the great simplification of experimental conditions which it provides. *In vitro* there are no vascular or nervous systems, and the influence of other organs is completely eliminated; thus any effect produced by a chemical agent *in vitro* must be due to its direct action on the tissue. Also the experimental conditions can be widely varied at will and are more easily controlled in a tissue culture than in the body; the technique has the further advantage that the living explants can be observed under the microscope whenever desired. It was therefore decided to undertake experiments on the effects of hypervitaminosis A on embryonic long bones cultivated *in vitro* by the watch-glass method.

In the living animal, hypervitaminosis A renders the long bones fragile, so that they sometimes suffer spontaneous fracture. This has often been observed in young rats fed on a diet containing an excessive quantity of vitamin A

(Collazo & Rodriguez, 1933; Bomskov & Seemann, 1933; Davies & Moore, 1934; Strauss, 1934; Van Metre, 1947; and many others). The same effect may also be produced by large doses of pure vitamin A (Wolbach & Bessey, 1942; Moore & Wang, 1943, 1945; Herbst, Pavcek & Elvehjem, 1944; Pavcek, Herbst & Elvehjem, 1945).

These gross effects of hypervitaminosis A are associated with drastic histological changes. Strauss (1934) observed retardation of osteogenesis, reduction of endochondral ossification and a variable degree of osteoclasis in the region of the fractures. Irving (1949) described rarefaction and reduced deposition of bone in the mandible of young rats. Wolbach & Bessey (1942) and Wolbach (1947) showed that in the limb bones of young rats and guinea-pigs the maturation and degeneration of the cartilage cells and the replacement of cartilage by bone were greatly accelerated and resulted in the abnormally early closure of the epiphyses. The fractures appeared to be due to 'the extensive loss of previously formed cortical bone before the newly deposited bone has acquired firmness sufficient to meet mechanical requirements' (Wolbach, 1947).

Whether the changes resulting from hypervitaminosis A were due to a direct action of the vitamin on the bone or to an indirect action through the endocrine glands, was uncertain. Recently, Barnicot (1950) has approached this problem by grafting experiments. Fragments of crystalline vitamin A acetate were attached to small pieces of parietal bone from 10-day mice and the combination was inserted into the cerebral hemispheres of litter-mates. When the grafts were removed 7–14 days later, advanced absorption accompanied by many osteoclasts was found, sometimes causing perforation of the bone. In Barnicot's experiments a direct action of vitamin A on the bone seems to have been clearly demonstrated, but the conditions were not wholly comparable with those in a hypervitaminotic animal. In the grafts the bone was in direct contact with a solid mass of vitamin, whereas in the hypervitaminotic animal it is only exposed to the relatively minute concentration present in the blood stream.

The experiments described below were undertaken to see whether the vitamin in concentrations similar to those in the blood of animals suffering from hypervitaminosis A had a direct effect on skeletal tissues and, if so, to learn something about the nature of its action. Two groups of experiments were made, one on the limb-bone rudiments from 5- to 6-day chick embryos to study the effect of hypervitaminosis A on the differentiation of bone and cartilage, and the other on late foetal mouse bones, to investigate the effect on bones which were already highly differentiated at the time of explantation. A comparison was also made of the effects of 'artificial' hypervitaminosis A produced in the culture medium by adding vitamin A alcohol or acetate to normal plasma, and of 'natural' hypervitaminosis, obtained by using plasma

drawn from a fowl suffering from hypervitaminosis A. A short preliminary account of the experiments on mouse bones has already been published (Fell & Mellanby, 1950).

TECHNIQUE

The explants

Chick. Chick embryos were removed from the egg after 5–6 days' incubation and the limb buds cut from the trunk. In the younger embryos (Exp. 48, Table 1) individual bone rudiments were not yet distinguishable and the axial core of procartilage was isolated and explanted whole: in the older embryos, the humerus, radius and ulna from the wing and the femur and tibia from the leg were explanted separately.

Mouse. Mouse bones were obtained from foetuses of about 17–20 days' gestation. The radius, ulna, tibia and fibula were cultivated; the humerus and femur were too bulky.

The culture medium

Embryo extract. The extract was made from chick embryos of 12–14 days' incubation. By drastic mincing and grinding, the embryo was reduced to a very fine, slimy pulp which was mixed with an equal volume of a modified Tyrode's solution (see below) and centrifuged for not more than 5 min. This produced a yellowish, rather turbid, fluid which was freshly prepared for each subculture.

The modified Tyrode's solution, mentioned above, contained 0·2 % calcium glycerophosphate and the glucose content was raised to 1 % for the experiments on chick bones and for Exp. 51 on mouse bones, and to 4 % for all the other experiments on mouse bones. In Exp. 2 the extract was made with ordinary Tyrode.

The vitamin A content of the embryo extract was estimated and found to be low, viz. 130 i.u./100 ml.

Preparation of plasma. The syringes were sterilized by heating in oil and were then cooled to 4° C. Three ml. of Ringer's solution containing 10 i.u. heparin/ml. was first drawn into the syringe after which blood was drawn from a wing vein so tha the volume was 30 ml. The contents of the syringe were gently mixed and then centrifuged, giving about 20 ml. of plasma.

'*Artificial*' *hypervitaminotic plasma* (*AH plasma*). This description refers to fowl plasma to which vitamin A had been added directly. In some experiments vitamin A alcohol was used and in others vitamin A acetate.

When vitamin A alcohol was added, a sample of avoleum containing 1,500,000 i.u. vitamin A/g. was dissolved in absolute alcohol to give a solution containing about 20,000 i.u./ml. The alcoholic solution was then added to normal plasma to raise the vitamin A content to the desired amount. The strength of the alcoholic solution of vitamin A was such, that the final plasma contained about 1 % ethanol in the earlier experiments and 0·2 % or less in the rest. When vitamin A acetate was added, the crystalline substance was dissolved in absolute alcohol to give amounts similar to those obtained with vitamin A alcohol. In each experiment, the same quantity of ethanol was added to the normal (control) plasma as to the AH plasma.

'*Natural*' *hypervitaminotic plasma* (*NH plasma*). This denotes plasma obtained from a fowl suffering from hypervitaminosis A.

All fowls were given a diet containing much cabbage, together with pellcts said to be sufficient for the normal feeding of these birds. In addition the hypervitaminosis A fowls were given 3 g. of avoleum by tube directly into their beaks (1 g. at each of the following times: 9 a.m., 2 p.m. and 5 p.m.) and 2 g. in the food. Each gram of avoleum contained 30,000 i.u. of vitamin A, so that each fowl was given 150,000 i.u. per diem.

At the end of a fortnight, or longer, the vitamin A supplement was stopped and the next morning the blood was withdrawn from the vein. In view of recent observations, the desired hypervitaminosis A might have been more rapidly obtained if the vitamin A oil had been first emulsified and it is also certain that a higher vitamin A content of the blood would have been obtained if it had been withdrawn on the same day as the last supplement of vitamin was given. The plasma of the normal bird contained about 200–400 i.u. of vitamin A and about 300 i.u. of

carotene per 100 ml., whereas that of the birds fed on large quantities of vitamin A contained about 1200–1500 i.u. of the vitamin and about 150 i.u. of carotene per 100 ml. Thus a high vitamin A diet usually lowered the carotene content of the plasma as compared with that of the normal bird, but raised its vitamin A content; a similar though less pronounced effect has been obtained in cattle (Walker, Thomson, Bartlett & Kon, 1949).

Estimation of vitamin A and carotene. The concentration of vitamin A and carotene in the plasma and embryo extract was estimated by a method closely similar to that of Yudkin (1941), a modification of Kimble's method (1939), and in detail was almost the same as that used by the Cambridge team in the investigation of the Medical Research Council (1949) on the vitamin A requirements of human adults. A sealed glass tube was used instead of a corked centrifuge tube when the plasma and alcohol mixture was extracted with petroleum ether. In the earlier experiments acetic anhydride was not added, as it was not necessary under the experimental conditions employed, but it was used in the later part of the work.

The final culture medium. The final culture medium consisted of 3 parts of fowl plasma mixed with 1 part of embryo extract. Thus the concentrations of calcium glycerophosphate and of glucose in the embryo extract were lowered to 25 % of their original values; the final medium contained 0·05 % of the former and 0·25 % glucose in the chick experiments and in Exp. 51 on mouse bones, and 1 % glucose in the other experiments on mouse bones. The high glucose content had a beneficial effect on the explants and reduced internal necrosis; the calcium glycerophosphate was added in the hope of improving ossification. It is probable that both these substances reached the interior of the bones in a much lower concentration than was present in the medium (cf. Trowell, 1952).

Since the vitamin A content of the embryo extract was low, the concentration of the vitamin in the final medium was about 25 % less than in the plasma. In the following account, the concentrations given are those estimated in the original plasma samples, so that the final medium contained about 25 % less vitamin A than the figure stated.

The vitamin A content of normal mouse plasma is only 20–60 i.u./100 ml., while that of fowl plasma is about 200–300 i.u./100 ml. Even the normal culture medium was therefore somewhat hypervitaminotic for the mouse bones.

Cultivation by the watch-glass method

In the watch-glass method (Fell & Robison, 1929; Fell, 1951) the culture vessel consisted of a watch-glass enclosed in a Petri dish which was carpeted with cotton-wool soaked with distilled water; a hole was cut in the cotton-wool to allow transillumination. First the plasma and then the embryo extract was dropped into the watch-glass, thoroughly mixed and spread with a fine glass rod and allowed to clot. The explants were placed in a drop of embryo extract made with ordinary Tyrode's solution, sucked into a pipette and deposited on the surface of the clot; all surplus fluid was removed from the tissue with a fine pipette. The watch-glass cultures were incubated on a strip of board, which prevented heavy condensation of water on the inner surface of the Petri dish lid.

The culture medium described above caused such rapid growth of the chick rudiments, that it was necessary to transplant them to freshly prepared watch-glasses four times a week; otherwise they exhausted the medium in their immediate neighbourhood and quite suddenly degenerated. For the same reason it was important to ensure that there was sufficient depth of medium beneath the rudiments; they were therefore explanted on 9 or 12 drops of medium, transferred to 15 drops at the first subculture and finally to 20 drops, the ratio of 3 parts plasma : 1 part embryo extract being maintained throughout. The modifications of culture medium and procedure adopted for the present experiments gave much better results than any previously obtained by H.B.F. when cultivating chick bone rudiments by the watch-glass technique.

The smaller and less actively growing mouse bones did well in 9–12 drops of culture medium and it was sufficient to transplant them three times a week. All the chick explants and the mouse bones in Exps. 51 and 52 were drawn with the aid of a camera lucida at the beginning of the experiment and at intervals during cultivation.

Histological technique

Most of the explants were fixed in acetic Zenker's fluid, embedded in paraffin wax and serially sectioned; in the later experiments with chick bone rudiments (section I) the bones were embedded in ester wax with very good results. The sections were usually stained with Delafield's haematoxylin and chromotrop 2 R. In the later experiments some sections were stained with 0·5% toluidine blue in 5% alcohol and van Gieson's stain preceded by celestine blue and Mayer's haemalum; in section III all the sections were stained with Nicolle's carbol thionin and counterstained with alcoholic eosin.

Some of the mouse bones were fixed in absolute alcohol and mounted whole in glycerin jelly, which gave a very clear general picture of their structure.

RESULTS

I. *The effect of cultivating the limb-bone rudiments of* 5- *to* 6-*day chick embryos in a medium containing excess vitamin A alcohol*

The object of these experiments was to study the effect of hypervitaminosis A on the differentiation of limb-bone rudiments which were at a very early stage of development when explanted.

The structure of the rudiments at the time of explantation. In the chick the rudiment of the limb skeleton is always slightly more developed in the leg than in the wing and the proximal elements are in advance of the more distal parts. The stage of differentiation may be clearly seen when the skeletal tissue is dissected under the binocular microscope.

There is considerable variation in the degree of development attained by embryos of the same age, and in the present study the embryos have been classified in four groups according to their stage of histogenesis:

Stage 1. The limb skeleton consisted of a continuous blastema of procartilage with no trace of a joint.

Stage 2. Individual bone rudiments were recognizable and were composed of very early cartilage in which chondroblastic hypertrophy had not yet appeared.

Stage 3. Chondroblastic hypertrophy was just distinguishable in the femur (Pl. 1, fig. 1) and humerus but not in the tibia, radius or ulna.

Stage 4. Chondroblastic hypertrophy was distinct in the femur and humerus, and had just appeared in the tibia, radius and ulna; a two-layered periosteum had differentiated round the middle segment of the shaft in the femur and humerus but the only sign of ossification was some fibres among the osteoblasts.

The behaviour of the rudiments in culture. The skeletal blastemas from limb buds at stage 1 (see above) were removed and cultivated entire, as the boundaries of individual bone rudiments were not yet distinguishable. The femur, tibia, humerus, radius and ulna of the older limbs were explanted separately.

Fifty-five pairs of explants were grown; one of each pair was cultivated in

normal medium and the other in the same medium to which excess vitamin A had been added. Explants were fixed and sectioned at intervals.

The results are summarized in Table 1. Only one experiment (Exp. 48) was done with blastemas at stage 1, a rather low concentration of vitamin A (1475 i.u./100 in plasma) being used. After 48 hr. growth, in both the control and experimental explants, the individual bone rudiments had become visible and the knee- and elbow-joints had appeared. In the two pairs of wing-buds, however, only the humerus developed well; it enlarged to several times its original length and acquired some semblance of its normal shape. There was little difference between the experimental and control humerus in the pair of wing explants fixed after 5 days; in both the cartilage differentiated into hypertrophic, proliferative and epiphysial zones and a thin layer of periosteal bone was deposited. In the 7-day pair, however, chondroblastic hypertrophy and ossification were much more advanced in the hypervitaminotic humerus than in the control. The radius and ulna in both pairs of wing blastemas grew much less than the humerus and underwent neither chondroblastic hypertrophy nor ossification.

The femur, tibia, fibula and part of the tarso-metatarsal region developed in the leg blastemas. The bones grown on the AH medium were rather more slender than those on the control medium. The femur, tibia and fibula of all four explants showed chondroblastic hypertrophy and ossification, but differentiation was more advanced in the hypervitaminotic rudiments than in the controls.

Control rudiments explanted at stages 2–4 grew and differentiated extremely well in cultures, sometimes increasing to more than four times their original length and assuming a surprisingly normal shape (Text-figs. 1 and 2). The soft tissue adhering to the cartilage proliferated rapidly and soon formed a capsule of organized connective tissue which often contained many muscle fibres and was permeated by a network of rather dilated endothelial channels. The zone of hypertrophic cartilage cells could be seen gradually spreading towards the ends of the diaphysis; the proliferative zone of flattened cells could be distinguished in life and the demarcation of this region from the epiphysis could also be observed. A yellowish layer of periosteal bone or osteoid was deposited on the surface of the hypertrophic cartilage, the structure of which it soon obscured. This layer became thicker and more opaque but it did not appear to become densely calcified. In the explants the cartilage enclosed by the bone was not excavated by ingrowing tissue as in normal development *in ovo*.

Sections of the control bones fixed after 9–11 days' cultivation in normal medium confirmed the observations on living cultures (Pl. 1, figs. 2, 4 and 6). The diaphysis consisted of hypertrophic cartilage enclosed by a thick sheath of periosteal bone; beyond the hypertrophic region was a broad proliferative zone of flattened cells which ended abruptly at the border of the small-celled

Table 1. The effect of vitamin A alcohol on the explanted limb-bone rudiments of 5- to 6-day chick embryos (AH, plasma with added vitamin A; N, normal plasma. Where two values are given for the vitamin A content, they refer to two samples of plasma used during the experiment.)

Exp.	Vitamin A (i.u./100 ml. plasma)	Stage of embryos (see p. 324)	Days in culture	No. pairs of bone rudiments	Effect of hypervitaminosis
49	AH: 902, 1050; N: 232, 410	2, 3	4	5: femora, tibiae, humeri, ulnae, radii	None; like controls in N medium
			11	5: femora, tibiae, humeri, ulnae, radii	Femur: smaller than control and cartilage matrix reduced. Tibia: looks normal but control lost. Humerus: like control. Ulna: much better developed than control. Radius: like control
48	AH: 1475; N: 334	1	5	6: femora, tibiae, fibulae, humeri, ulnae, radii	Femur, tibia, fibula: better developed than controls. Humerus, radius, ulna: like controls
			7	6: femora, tibiae, fibulae, humeri, ulnae, radii	Femur, tibia, fibula, humerus: better developed than controls. Radius, ulna: like controls
47	AH: 1640, 1550; N: 325, 450	4	7	5: femora, tibiae, humeri, ulnae, radii	Shorter and thinner than controls; constriction at one end of shaft in femur, tibia and humerus; in shaft cartilage matrix reduced and metachromasia less; good periosteal bone
			11	5: femora, tibiae, humeri, ulnae, radii	Smaller than controls, especially femur and humerus; constriction at both ends of shaft in femur and proximal end in humerus; metachromasia nearly gone from shaft; less periosteal bone than in controls. Note: in the control radius the metachromasia has gone from a short middle section of the shaft (only control to show this)
46	AH: 1980, 1920; N: 231, 167	2	7	2: femora, tibiae	Constriction at one end of tibia and both ends of femur; cartilage matrix losing basophilia in shaft; bone as good as in controls
			9	5: femora, tibiae, humeri, ulnae, radii	Proximal end of humerus severed; constriction of shaft at one end of tibia and both ends of femur; basophilia and metachromasia of cartilage matrix greatly reduced in shaft; thick periosteal bone
			11	3: humeri, ulnae, radii	Proximal end of humerus severed and displaced; metachromasia and basophilia gone from shaft; slightly less bone than in controls
43	AH: 2280, 2154; N: 212, 256	3	4	5: femora, tibiae, humeri, ulnae, radii	Constriction of shaft at both ends of femur and one end of tibia and humerus; cartilage matrix still basophilic except at margin of constrictions; rather more bone than in controls
			7	4: femora, tibiae, ulnae, radii	Femur: distal end severed, proximal end nearly gone; constriction at one end of tibia; basophilia nearly gone from shaft in femur and tibia, partly gone in ulna, unchanged in radius; rather less bone than in controls
50	AH: 2800, 3247; N: 290, 260	3	7	5: femora, tibiae, humeri, ulnae, radii	Constrictions at both ends of femur, tibia, humerus; basophilia and metachromasia lost from periphery of shaft; rather less bone than in controls
		2	11	5: femora, tibiae, humeri, ulnae, radii	Constrictions at both ends of all bones except tibia; basophilia and metachromasia gone except in interior of epiphyses; less bone than in controls

cartilage of the epiphysis. There were many mitotic figures in the epiphyses and proliferative zones. The cartilage matrix was densely basophilic when stained with Delafield's haematoxylin and acquired an intense metachromatic coloration with toluidine blue (Pl. 1, figs. 4, 10; Pl. 2, fig. 12); it remained almost colourless after van Gieson's stain. Especially in the humerus, there was sometimes an area of mucoid degeneration in the interior of the broad terminal regions of the shaft; the matrix was swollen, showed disintegration and the cells, though not dead, were widely separated by the swollen matrix. Such appearances were probably due to imperfect nutrition in the centre of the large, avascular explants.

The periosteal bone, which was vividly red after van Gieson's stain, was slightly metachromatic with toluidine blue. Where the ossified layer was very thick, small islets of cartilage were sometimes present, merging with the surrounding bone; in such specimens the histological picture somewhat resembled that of callus in which cartilage and bone are irregularly mingled. Metachromasia has been described in normal osteoid tissue by Follis & Berthrong (1949).

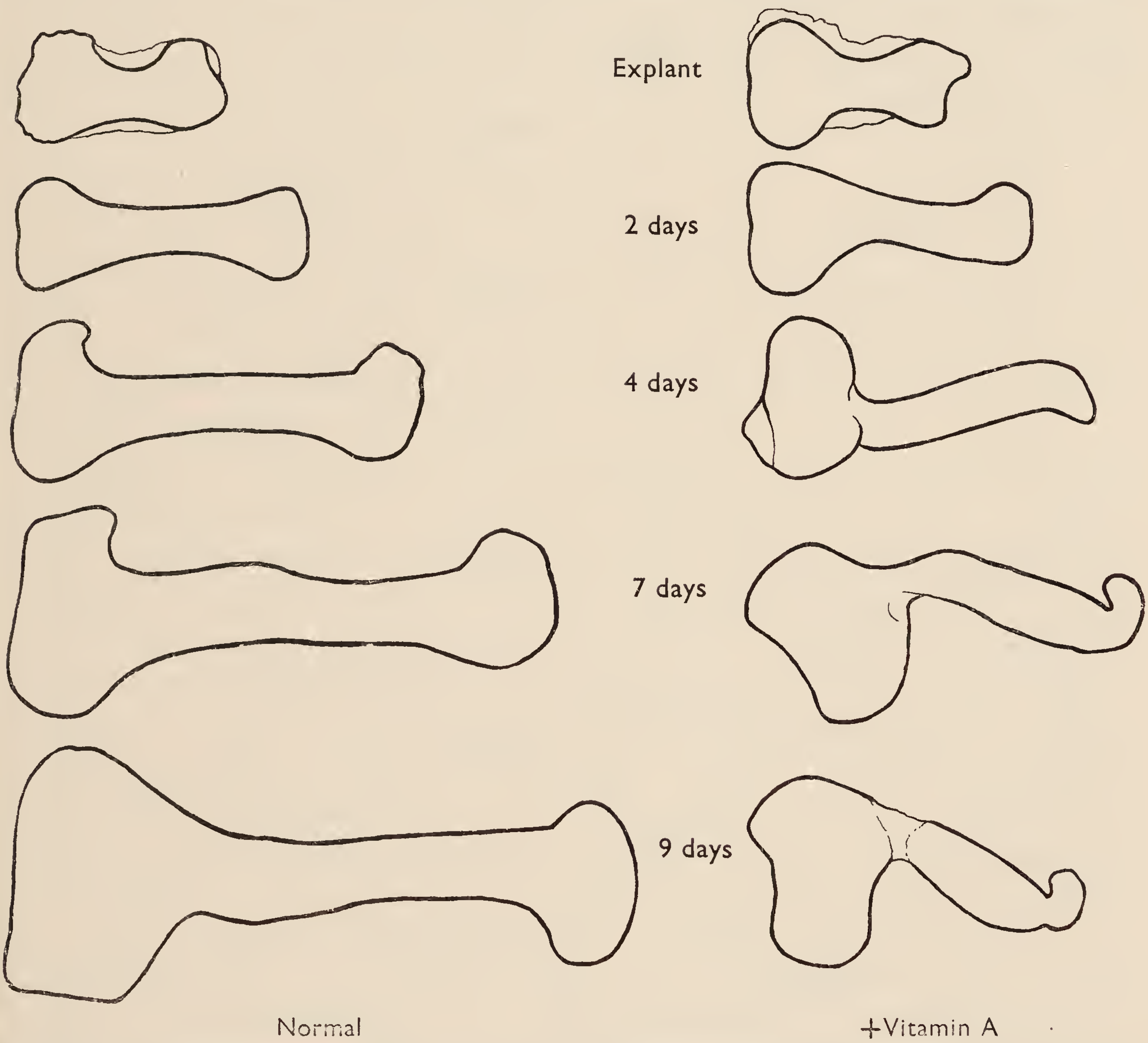
The hypervitaminotic explants of stages 2–4 were grown in concentrations of vitamin A ranging from 902 to 3247 i.u./100 ml. plasma. The lowest concentration (Exp. 49, Table 1) produced little change, and after 4 days' cultivation the experimental and control rudiments were indistinguishable. In the 11-day cultures the hypervitaminotic femur was considerably smaller than its control in normal medium, and sections showed that the intercellular partitions in the cartilage were much thinner. Chondroblastic hypertrophy and ossification were much more advanced in the hypervitaminotic ulna than in the control, the development of which was somewhat retarded. There was no obvious difference between the other experimental and control explants.

In concentrations of about 1550–3247 i.u. vitamin A/100 ml. of plasma (Text-figs. 1, 2), the rudiments grew normally for the first two days and did not differ from their controls. Then growth began to decline; the rate of elongation diminished and the shaft ceased to broaden, so that it became both shorter and more slender than in the controls. The epiphyses continued to enlarge but less than in the normal cultures. Eventually growth stopped and some of the explants even began to shrink. Periosteal ossification progressed and the differentiation of the hypertrophic cartilage, zones of flattened cells and epiphyses could be watched.

In some of the hypervitaminotic explants, at about the 7th day a constriction appeared in the zone of flattened cells (proliferating zone) just where the sheath of periosteal bone ended (Text-fig. 2). This was most conspicuous at the proximal end of the humerus and at the distal end of the femur; later a constriction usually appeared also at the proximal end of the femur and sometimes, though to a lesser degree, at the distal end of the tibia. The terminal

part of the rudiment beyond the constriction often became completely detached from the shaft and was sometimes displaced to one side (Text-fig. 2). In the highest concentration (Table 1, Exp. 50) by the 11th day a constriction had formed at both ends of all the bones except the tibia.

The consistency of the hypervitaminotic explants changed during cultivation. Instead of remaining firm and stiff like the controls, when transferred

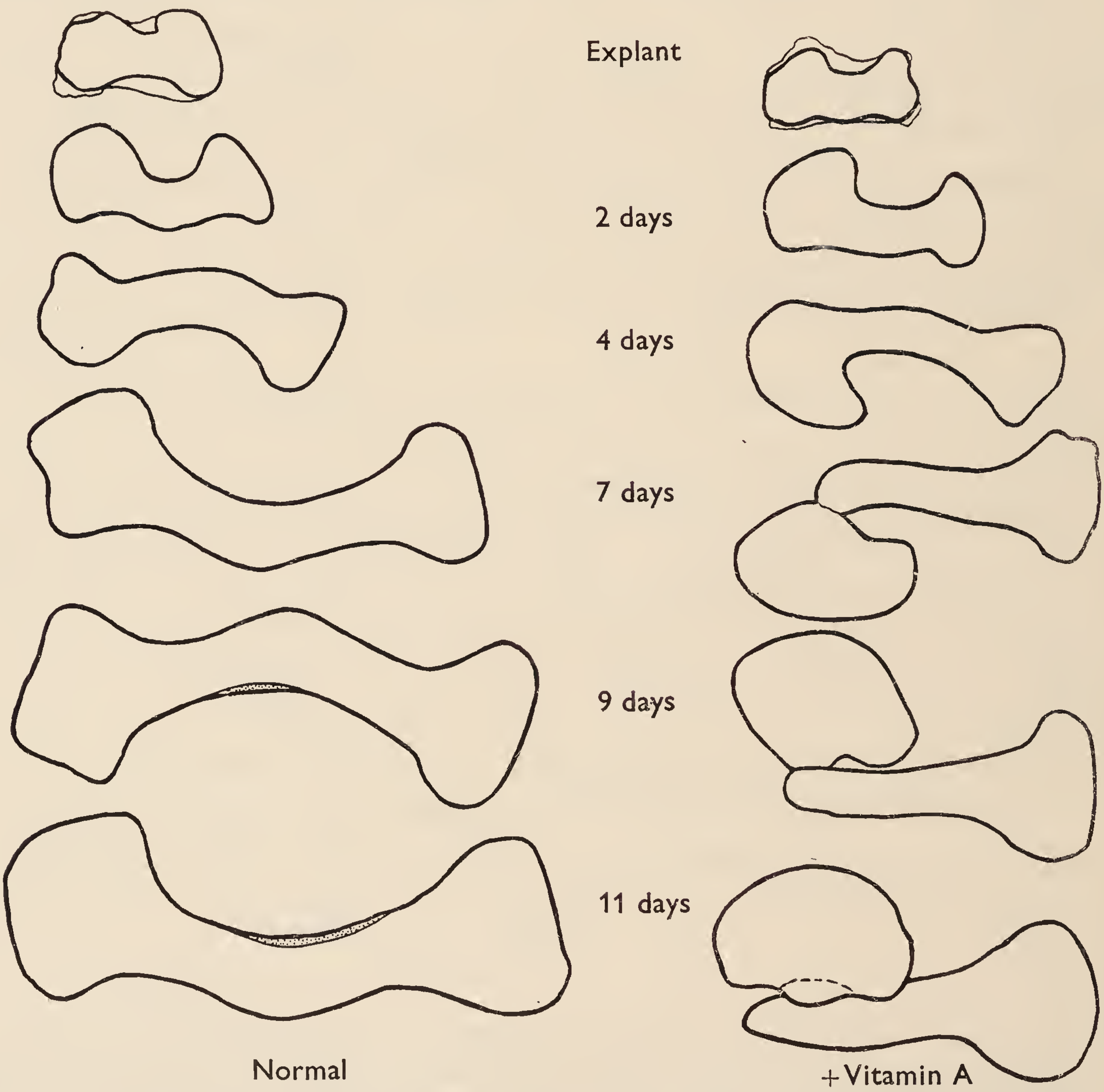


Text-fig. 1. Camera lucida drawings of a pair of living femora from a 6-day chick embryo (stage 2) one grown in normal and the other in AH medium (Exp. 46, Table 1). During 9 days' cultivation the femur in normal medium elongated from 1·6 to 5·2 mm. and showed no distortion. That in AH medium grew normally for the first 2 days; then growth rapidly declined, a constriction appeared in the distal part of the shaft and by the 9th day the condylar end was nearly detached from the diaphysis and the rudiment was shrunken and bent. Sections of these explants are shown in Pl. 1, figs. 2 and 3.

to fresh medium they were found to be soft and 'rubbery'. Throughout the culture period the cells surrounding the cartilage grew profusely, forming a thick capsule of connective and myogenic tissue around the skeletal rudiment.

Sections of the explants grown in the higher concentrations of vitamin A revealed some interesting histological abnormalities. The differentiation of the

cartilage into epiphyses, zones of flattened cells and hypertrophic region was at least as rapid as in the controls and in two explants (Exp. 49, ulna, and Exp. 50, radius) it was much more advanced. The layer of periosteal bone deposited on the surface of the cartilage was in some explants nearly or quite as thick as in the controls (Pl. 1, cf. figs. 4 and 5); in the highest concentration of vitamin A (Exp. 50) many of the osteoblasts in some of the larger rudiments were degenerate by the 11th day of cultivation, but in other explants the majority remained healthy throughout the culture period.



Text-fig. 2. Camera lucida drawings of living humeri from a 6-day chick embryo (stage 2), one grown in normal and the other in AH medium (Exp. 46, Table 1). During 11 days' cultivation, the humerus in normal medium elongated from 1·3 to 5·2 mm. and developed a fairly normal shape. That in AH medium also enlarged considerably, but a constriction appeared at the proximal end of the shaft and by the 9th day the proximal epiphysis had become completely detached from the diaphysis. Sections of these explants are shown in Pl. 1, figs. 6 and 7.

The most striking histological effect of the hypervitaminosis was on the cartilage matrix, and to this were due the severe changes described above in the living cultures. The intercellular partitions began first to narrow and then to lose their metachromasia (Pl. 2, cf. figs. 11 and 12); under high magnification the fibrous texture demonstrated by staining with toluidine blue was seen

to be looser than in the controls. The loss of metachromasia spread inwards from the periphery (Pl. 1, figs. 9 and 10) until by about the 11th day the matrix of the whole shaft was completely colourless when stained with Delafield's haematoxylin or toluidine blue (Pl. 1, cf. figs. 4 and 5; Pl. 2, fig. 13). When neighbouring sections of the same hypervitaminotic explant were stained with van Gieson's solution and with toluidine blue respectively, it was found that in those areas where the matrix had lost its metachromasia with toluidine blue, it stained a bright pink with van Gieson's solution; conversely, regions which still retained their metachromasia were nearly colourless after van Gieson's stain, as also was the cartilage of the controls grown in normal medium (Pl. 2, cf. figs. 13 and 14).

In only one of the fifty-four controls grown in normal medium was there a similar loss of metachromasia in the cartilage matrix. This was seen in a control radius after 11 days' cultivation (Exp. 47); a short segment in the middle of the shaft failed to stain metachromatically with toluidine blue; in the corresponding radius grown in AH medium the metachromasia had almost completely disappeared from the whole diaphysis.

The matrix of the epiphyses was less affected by the hypervitaminosis than that of the shaft, but after 11 days' cultivation in the highest concentration of vitamin A (Exp. 50), the metachromasia persisted only in small regions in the interior of the epiphyses and elsewhere had vanished completely (Pl. 2, fig. 13).

While these drastic changes were progressing in the matrix, the chondroblasts throughout most of the affected cartilage appeared healthy (Pl. 1, fig. 5 and Pl. 2, fig. 11), though in the hypertrophic region of the shaft they became smaller and less vacuolated than in the controls (Pl. 1, cf. figs. 4 and 5). In some of the 11-day explants grown in the highest concentration of vitamin A (Exp. 50), the intercellular partitions had disappeared completely in places, so that groups or nests of cells were often enclosed in one large capsule; in some explants it was surprising to find many of these cells in mitosis.

In those hypervitaminotic explants in which, as already mentioned, a constriction formed near the end of the diaphysis (Text-fig. 2), localized necrosis was always present at this site, where the following events took place. Immediately beyond the sheath of periosteal bone, the peripheral cartilage matrix lost its metachromasia, softened and shrank in the usual way; the chondroblasts became closely packed together and some degenerated, while the boundary between the cartilage and the surrounding connective tissue became indistinct. As the changes in the matrix spread inwards, a tract of cartilage extending right across the shaft began to show pressure effects (Pl. 1, fig. 8): the cells became greatly flattened and some degenerated, while the matrix was compressed into fibrillae staining brightly with van Gieson's stain or chromatrop and orientated at right angles to the long axis of the rudiment (Pl. 2, fig. 14). The pressure responsible for these changes was apparently exerted by the capsule of myogenic and connective tissue which enveloped

the cartilage and which seemed to contract when the tissue was removed from the clot. The zone of cartilage just beyond the periosteal bone was probably more susceptible to pressure than the rest of the rudiment, since it was presumably the weakest part; it was narrower than the terminal part of the diaphysis which broadened rapidly on approaching its junction with the epiphysis, and lacked the support of the stiff sheath of bone which reinforced the rest of the diaphysis.

The necrotic layer of cartilage produced in this way was sometimes invaded by the adjacent connective tissue cells, until the epiphysis and the wide terminal part of the zone of flattened cells was completely severed from the ossified shaft. Continuing pressure often bent the detached end to one side and occasionally forced it down alongside the shaft. The periosteal bone might grow over the broken end of the diaphysis and in one explant (Pl. 1, fig. 7) ossification spread diffusely into the surrounding connective tissue.

From these results it is concluded that:

(1) The cartilaginous long-bone rudiments of 5- to 6-day chick embryos differentiate at the normal rate into epiphyses, zones of flattened cells and hypertrophic region when cultivated in medium to which excess vitamin A has been added.

(2) Periosteal ossification of the long-bone rudiments is only slightly inhibited by hypervitaminosis A.

(3) Hypervitaminosis A produces drastic changes in the cartilage matrix of the explants, viz. shrinkage of the intercellular partitions, softening and loss of basophilia and metachromasia.

(4) These changes are not associated with degeneration of the chondroblasts.

II. *The effect of cultivating late foetal and early infant mouse bones in normal medium containing excess vitamin A acetate or alcohol*

These experiments were made to study the effect of hypervitaminosis A on bones that were already at an advanced stage of development when explanted.

The structure of the bones at the time of explantation. In every experiment on late *foetal mouse bones*, a fore- and hind-limb of one foetus from each litter was fixed and sectioned to show the approximate stage of development of the original explants. The late foetal bones were well developed (Pl. 2, fig. 15). Most of the shaft consisted of a tube of trabecular bone covered by a periosteum with an outer fibrous and an inner osteoblastic layer. The cartilage had been completely excavated from most of the diaphysis and replaced by marrow, permeated by trabeculae of endochondral bone, but the ends of the shaft (metaphyses) were largely occupied by hypertrophic cartilage undergoing endochondral ossification and separated from the round-celled epiphyses by broad tracts of flattened cells with the usual columnar arrangement; there was no sharp demarcation between the three zones of cartilage. The cartilage

matrix was intensely basophilic with Delafield's haematoxylin and strongly metachromatic with toluidine blue; both stains showed clearly the cartilaginous centres of the endochondral bone trabeculae.

The bones used for Exp. 51 (Table 2) were slightly younger than the rest. The marrow cavity occupied only about one-third of the length of the shaft, and the periosteal bone was more cellular and less abundant than in the older rudiments. Otherwise the histological structure was similar to that described above.

The normal bones of 3-*day infant mice* were not taken from the same litter as that which provided the explants. The periosteal bone was much less trabecular and more compact than in the foetal rudiments and little endochondral bone remained in the middle segment of the shaft. The strongly basophilic and metachromatic terminal cartilage occupied a smaller proportion of the shaft than in the late foetus, but otherwise was similar. The articular cartilage, however, was better developed; in some regions it was much less metachromatic with toluidine blue than the rest of the epiphysial cartilage and stained brightly red with van Gieson's solution.

The behaviour of the bones in culture. Fifty pairs of foetal bones and four pairs of infant mouse bones were used in these experiments. Of each pair, one was grown in medium to which an alcoholic solution of vitamin A acetate or alcohol had been added to give various concentrations, the other in the same medium containing the same quantity of ethanol but no added vitamin A.

During cultivation in normal medium, the control *foetal bones* elongated (Text-fig. 3), but much less than the early skeletal rudiments of the chick, and the cartilaginous ends broadened. The cells from the soft tissue attached to the bone wandered into the clotted medium and by the 3rd day had formed a broad zone of outgrowth which anchored the bone to the clot and thus resisted its further expansion; this sometimes forced the cartilaginous ends slightly out of alinement with the shaft in 7- to 10-day cultures.

In sections (Pl. 2, figs. 16 and 18), surprisingly little cell degeneration was seen in the controls even after 10 days in culture, the longest period for which the explants were maintained, but they showed certain abnormalities which were probably due partly to the lack of a vascular system and partly to the fact that, as mentioned above, normal fowl plasma is somewhat hypervitaminotic for mouse bones.

As in normal development, the periosteal bone was partially reorganized to become less trabecular and more compact (Pl. 2, cf. figs. 15 and 16). Sometimes layers of new bone were deposited on the outside of the diaphysis parallel with the surface, so that the wall of the shaft was thickened. In other explants, especially in those of Exp. 51 which were obtained from rather younger embryos than the rest, bone absorption slightly exceeded bone deposition; as will be seen later, the younger bones were more sensitive to the action of vitamin A than the older rudiments, so that this slight rarefaction of

Table 2. The effect of vitamin A acetate and alcohol on foetal mouse bones
(AH, plasma with added vitamin A; N, normal plasma)

Exp.	Vitamin A (i.u./100 ml. plasma)	Days in culture	No. pairs of bone rudiments	Effect of hypervitaminosis
11	AH: 1000	7	2: radii, ulnae	Cartilage much reduced; matrix still basophilic; bone rarified
	(vit. A acetate) N: 220	10	4: tibiae, fibulae, radii, ulnae	Cartilage nearly gone; little or no basophilia in matrix; bone very rarified and nearly gone in fibula
12	AH: 1080	3	2: tibiae, fibulae	Like controls
	960	7	2: radii, ulnae	Cartilage reduced; basophilia of matrix nearly gone; bone rarified
	(vit. A acetate) N: 382 400	10	2: radii, ulnae	Cartilage much reduced; very little basophilia in matrix; extensive bone absorption
	AH: 1880	3	2: tibiae, fibulae	Cartilage reduced; partial loss of basophilia in matrix; bone unchanged
	1920	5	2: tibiae, fibulae	Cartilage much reduced, especially in fibula; bone much eroded in tibia, nearly gone in fibula
		7	2: radii, ulnae	Cartilage nearly (ulna) or completely gone (radius); great absorption of bone
	AH: 2640	3	2: tibiae, fibulae	Cartilage much reduced; partial loss of basophilia in matrix; some bone absorption
	2720	7	2: radii, ulnae	Cartilage gone; very great bone absorption
52	AH: 2845 (vit. A alc.)	4	4: tibiae, fibulae radii, ulnae	Cartilage much reduced; metachromasia nearly or completely gone; bone greatly rarified
	N: 261	7	4: tibiae, fibulae radii, ulnae	Cartilage nearly (tibia) or completely gone; great absorption of bone
51	AH: 2876 (vit. A alc.)	2	4: tibiae, fibulae, radii, ulnae	Cartilage much reduced; partial loss of metachromasia in matrix; extensive bone absorption with many osteoclasts
	N: 268	4	4: tibiae, fibulae, radii, ulnae	Cartilage greatly reduced; metachromasia almost or completely gone from matrix; bone nearly gone. Some rarefaction of bone in controls
7	AH: 4000 (approx.)	7	2: tibiae, fibulae	Cartilage nearly gone; metachromasia gone in matrix; great bone absorption
	(vit. A acetate) N: unknown	10	2: radii, ulnae	Cartilage gone; bone almost gone
2	AH: 8000 (approx.)	5	2: radii, ulnae	Cartilage greatly reduced; basophilia gone from matrix; much bone absorption
	(vit. A acetate) N: unknown	8	2: tibiae, fibulae	Cartilage greatly reduced (tibia) or gone (fibula); basophilia nearly gone from matrix in tibia; extensive bone absorption

the periosteal bone in the controls may have been due to the hypervitaminosis A of normal fowl medium for mouse bones. Relatively few osteoblasts and osteocytes were degenerate except in the innermost periosteal trabeculae of the larger bones (tibia and sometimes ulna) and in the endochondral spongy bone.

As in the normally developed limb bones, there was considerable absorption of the bone trabeculae in the marrow cavity, but in the explants endochondral ossification was almost arrested, so that the spongy bone of the metaphyses was not replaced as it is in normal development (Pl. 2, fig. 16). Instead, the hypertrophic cartilage was slowly invaded and excavated by cells from the marrow; erosion was sometimes very irregular and sometimes proceeded along a fairly straight front. In some explants a layer of osteoid tissue was deposited on the surface of the cartilage which was thus sealed from the marrow cavity, the histological picture somewhat resembling that of the terminal plate of bone formed on the surface of the epiphysial cartilage during arrested growth *in vivo*, e.g. in hypophysectomized young rats (Ray, Evans & Becks, 1942). This removal of the pre-existing spongy bone and failure to replace it by further endochondral ossification, weakened the metaphyses which, as described above, sometimes bent or crumpled slightly owing to the resistance to elongation caused by the outgrowth of cells into the clot; for the same reason the metaphyses often became abnormally narrow, so that the terminal cartilage bulged rather abruptly from the bony shaft.

The cartilage matrix continued to increase in amount and the intercellular partitions broadened. With Delafield's haematoxylin it was strongly basophilic; with toluidine blue (used in Exps. 51, 52 only) it was intensely metachromatic (Pl. 2, fig. 18), except in the near neighbourhood of the excavation at the marrow surface, where the staining capacity was lost or greatly reduced. After van Gieson's stain (Exps. 51, 52 only), the matrix of the hypertrophic cartilage was pale pink as in normal development; elsewhere in the explants of Exp. 52 it was nearly colourless and pale pink in those of Exp. 51 for which, as already mentioned, rather younger rudiments had been used.

The chondroblasts remained apparently healthy throughout, except in the hypertrophic cartilage most deeply situated in the bony shaft where, especially in the relatively large tibiae, many or all of the cells were dead by the 7th day; in normal development this region would already have been excavated by the ingrowing vessels and associated tissues. The proliferative zone showed signs of diminishing activity; the columnar arrangement of the chondroblasts became disorganized and the cells less flattened so that by the 10th day the zone could hardly be distinguished from the adjacent cartilage.

Most of the haemopoietic cells originally present in the marrow degenerated during the first few days in culture; their remains were phagocyted and, in the smaller bones, were often removed completely. After the destruction of

the original marrow, haemopoiesis continued, especially in the smaller explants, though on a greatly reduced scale; most of the marrow cavity was occupied by the reticulum, a loose tissue of irregularly arranged fibres and cells some of which were in mitosis; there was also a network of thin-walled vessels. A few osteoclasts were always found, they were more numerous in the controls of Exp. 51 than in the controls of the other experiments.

From the foregoing account, it will be seen that in normal fowl medium, the control foetal mouse bones survived with little necrosis for at least 10 days and underwent some further differentiation, but that their growth was at first greatly retarded and finally arrested by conditions *in vitro*.

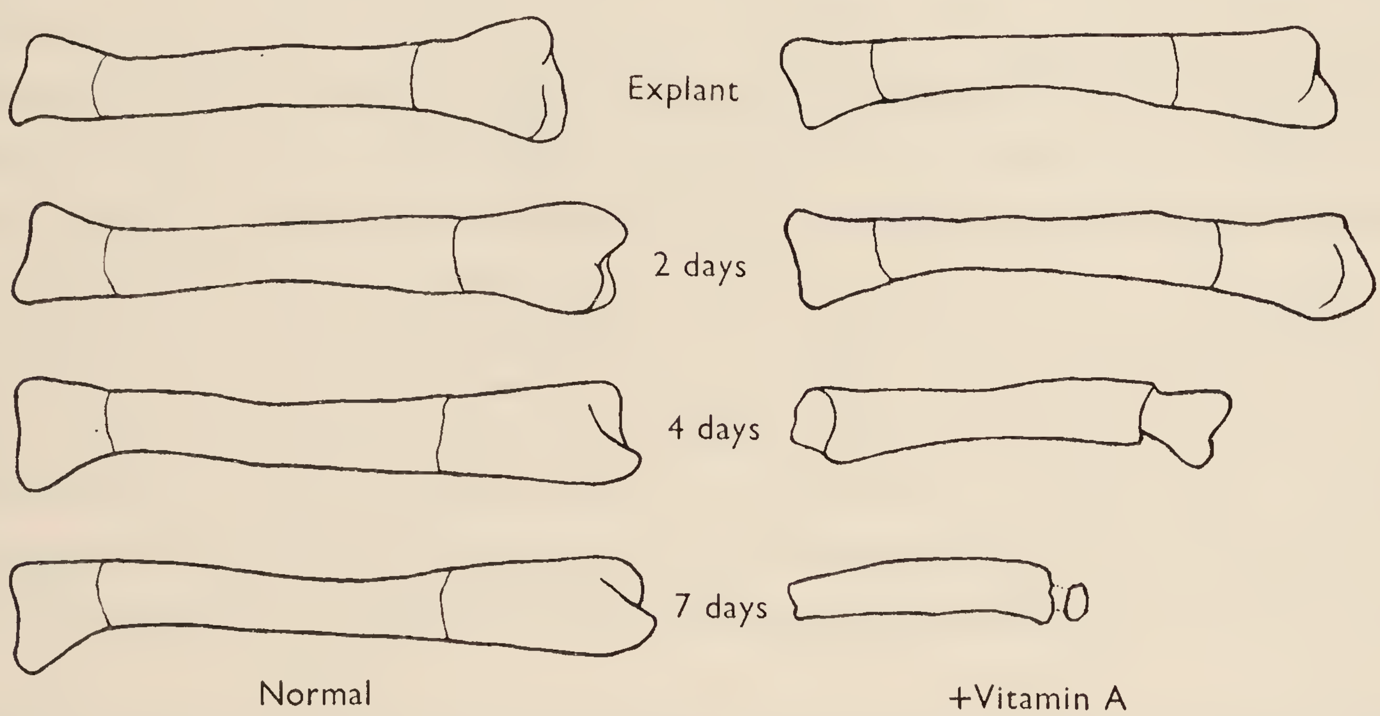
The effect of hypervitaminosis A on the corresponding bone rudiments cultivated in medium containing excess vitamin A acetate or alcohol, was remarkable. It was most conspicuous in the explants of Exp. 51 (Table 2), for which younger embryos were used than for the other experiments. These rudiments were grown in a concentration of 2876 i.u. vitamin A/100 ml. plasma and after only 2 days *in vitro* they had shrunk considerably, while the controls had enlarged during this period. When the living cultures were examined under the dissecting binocular, the bone was seen to be greatly rarefied and the cartilaginous ends much reduced in size as compared with the corresponding controls. After 4 days, when the experiment was terminated, the bone had nearly disappeared from the hypervitaminotic rudiments, only a few small spicules remaining, while the cartilage had dwindled to a tiny nodule at either end of the original shaft region; the controls showed little change except that, as mentioned above, the diaphysial bone was rather less dense than at the beginning of the experiment. The soft tissue surrounding the rudiments grew with equal vigour in both series.

In the other experiments on rather more advanced bones, the cartilage disappeared more quickly than the shaft. Except at the highest concentrations (Exps. 2 and 7, Table 2), little difference was seen between the hypervitaminotic and control explants during the first 2 days in culture (Text-fig. 3), but after 4 days there was a marked contrast between them. The explants in excess vitamin A were much smaller than the controls; the terminal cartilage which looked rather opaque, was shrunken and often displaced at an angle to the bony shaft in which jagged holes had appeared.

The cartilage diminished very quickly and soon ceased to fit the diaphysial bone, from which it emerged like a small head and neck from a too large collar (Text-fig. 3); sometimes it was forced downwards into the marrow cavity by the pressure of the surrounding tissue. Meanwhile, the periosteal bone continued to be absorbed rapidly; by the 5th day it had a moth-eaten appearance and had become much shorter. Different rudiments reacted at different rates; the changes were most advanced in the fibula, less in the radius, less still in the ulna and least in the tibia.

During the later stages of cultivation the shaft became greatly disorganized (Text-fig. 3); by the 10th day the cartilage had often disappeared in the fibula and radius and the bone had disintegrated. All that remained of the original explant was an actively growing sheet of amoeboid cells in which were scattered a few spicules of bone. In the tibia small nodules of cartilage and a little bone persisted.

Histological examination of the foetal bones grown in AH medium showed that the disappearance of the cartilage was due to two causes: a progressive narrowing of the partitions of matrix between the chondroblasts and an abnormally fast excavation at the marrow surface. As in the chick rudiments,



Text-fig. 3. Camera lucida drawings of living radii from a late mouse foetus, one grown in normal and the other in AH medium (Exp. 52, Table 2). During 7 days' cultivation the radius in normal medium enlarged somewhat and the bone and cartilage remained intact. That grown in AH medium also enlarged during the first 2 days, then suddenly the cartilage began to shrink and the bone to be absorbed; by the 4th day the cartilage had almost disappeared and the bone was much rarefied and reduced. Sections of these explants are shown in Pl. 2, figs. 15–19.

shrinkage of the matrix was accompanied by loss of basophilia and metachromasia, which began at the surface and spread inwards (Pl. 2, fig. 21), and those areas which had lost their metachromasia stained more brightly with van Gieson's solution.

During the original dissection, small superficial areas of cartilage were usually killed by pressure when the cataract knife was inserted between adjacent articular surfaces to isolate the bones. In the controls grown in normal medium, these little necrotic patches formed a negligible part of the terminal cartilage and were hardly noticeable. In the hypervitaminotic explants, however, they became increasingly conspicuous, as the matrix did not shrink and lose its basophilia in these killed regions nearly so fast as in the neighbouring undamaged cartilage where the cells were still alive; this indi-

cated that the chondroblasts were themselves concerned in the destruction of their intercellular material.

At the marrow surface large groups of liberated hypertrophic chondroblasts were sometimes seen during the first few days of cultivation.

How the bone was absorbed was not entirely clear from the sections. In Exp. 51 the number of osteoclasts was considerably greater in the hypervitaminotic bones, but in other experiments there seemed to be no more than in the controls, and the appearance suggested that the other cell types, e.g. macrophages, reticular cells, endothelial cells and possibly the osteoblasts and osteocytes, were responsible for the absorption.

There was little, if any, deposition of new osteoid tissue in the hypervitaminotic mouse bones, which in this respect differed from those of the 6-day chick embryos in which ossification was nearly as active as in the controls. Nevertheless, the osteoblasts in the hypervitaminotic mouse explants multiplied actively so that the tissue around the bone became much denser than in the controls (Pl. 2, cf. figs. 16 and 17) and contained many mitotic figures. It is possible that more growth-promoting material from the culture medium reached the interior of the shaft as the periosteal bone was eroded, and encouraged the cells to multiply; later the general shrinkage of the shaft probably compressed the cells and so further increased their density. The osteoblasts soon lost their characteristic appearance, however, and became transformed into spindle-shaped cells of the fibroblast type which continued to divide.

Sections showed that in the final stages of the explants' dissolution, the cartilage had shrunk to a compact mass of chondroblasts enclosed by very thin capsules which stained pink with van Gieson's solution or chromotrop. Eventually these capsules also disappeared, leaving the cells free. The subsequent fate of the chondroblasts depended on the amount of tissue by which they were surrounded. If tags of muscle and tendon had been left attached to the original cartilage, the cells proliferated and enveloped the cartilage by a fairly thick layer of connective tissue; when the matrix had gone, the soft mass of cartilage cells was compressed by this surrounding layer and degenerated. On the other hand, if the cartilage had been dissected clean, all but the innermost chondroblasts in the larger rudiments and nearly all those in the smaller explants survived the loss of their capsules and seemed to wander into the medium where they were lost (Pl. 2, fig. 22).

In Exp. 51 in which, as already described, the bone was absorbed with extraordinary speed, the osteoblasts which at first multiplied profusely and showed very active mitosis, became greatly compressed by the very rapid shrinkage of the diaphysis and most of them degenerated. The destruction of the shaft was more gradual in the explants from older foetuses and there was little degeneration among the cells of the marrow cavity; as the bone vanished,

these cells spread out on the medium and continued to multiply. Finally all that was left of the explants (Pl. 2, fig. 20) was a mass of fibroblasts and macrophages with a few shreds of bone and one or more of the small islets of dead cartilage mentioned above.

The rate at which the explanted foetal bones reacted to hypervitaminosis A depended on the concentration of the vitamin in the medium. Thus, as may be seen from Table 2, Exp. 12, after 3 days' cultivation, a tibia explanted in medium containing about 1000 i.u. vitamin A/100 ml. of plasma showed no change, in about 2000 i.u./100 ml. the cartilage had begun to shrink and lose its basophilia and in about 3000 i.u./100 ml. the cartilage was greatly reduced, had lost much of its basophilia and the bone was partly absorbed. Similarly, after 7 days' cultivation, a radius in medium containing about 1000 i.u. vitamin A/100 ml. plasma retained some cartilage which, however, was greatly reduced and had nearly lost its basophilia, and the shaft persisted, though much rarefied, while at a concentration of about 3000 i.u./100 ml. the rudiment had almost vanished.

The results of the experiment on radii and ulnae from 3-*day infant mice*, cultivated in medium containing 3316 and 3180 i.u. vitamin A/100 ml. plasma, were similar to but less drastic than those described above. The controls explanted in normal medium grew little and endochondral ossification was arrested; a terminal plate of bone, like that described on p. 334, was deposited on the surface of the hypertrophic cartilage at the proximal end of the radius. The marrow degenerated, but even after 10 days' cultivation there were few necrotic cells, in spite of the large size of the explants and the density of their intercellular material.

In the hypervitaminotic bones the cartilaginous ends showed severe changes after only 5 days' cultivation; they were much smaller than those of the controls and in the radius the matrix had lost all and in the ulna nearly all its basophilia with Delafield's haematoxylin. The chondroblasts appeared healthy. The diaphysial bone was thinner than in the controls and the osteoblasts were fewer and less well developed. After 10 days the cartilage had nearly gone and the periosteal bone was extensively absorbed, especially near the ends of the diaphysis.

It may be concluded that *in vitro*:

(1) Hypervitaminosis A causes the cartilage matrix to shrink, lose its basophilia and metachromasia and finally to disappear.

(2) The chondroblasts of the affected cartilage appear healthy.

(3) There is great absorption of bone.

(4) The younger the bone rudiment when explanted, the greater is its susceptibility.

III. *The effect of heating* (a) *the bones,* (b) *the plasma of the culture medium on the growth of foetal mouse bones in normal medium and in medium containing excess vitamin A alcohol*

In the previous section it was shown that areas of cartilage which had been killed by mechanical damage did not respond to hypervitaminosis A like the adjacent, living cartilage. The following experiments were made to see what relationship, if any, existed between the growth of bones *in vitro* and their response to hypervitaminosis A.

The effect of heating the embryos. Mouse embryos, after removal of one fore-paw, were placed in a tube containing about 20 ml. of Ringer's solution and a thermometer. The tube was then immersed in a bath at a temperature slightly higher than that desired for the heating of the embryo. When the required temperature of 60, 50, 45, 40 or 37° C. had been reached, it was maintained for 15 min., after which the tube was quickly cooled to room temperature. The radius and ulna were dissected out of both fore-paws and explanted on the culture medium in the usual way. The explants were transferred to fresh medium on the 2nd day and fixed on the 4th. To produce the hypervitaminotic medium about 2280 i.u. vitamin A alcohol/100 ml. was added to the plasma.

The reaction of the rudiments to different temperatures was found to be delicately balanced. Thus rudiments from embryos heated to 60, 50 and 45° C. respectively for 15 min. failed to grow on normal medium, and sections showed that after 4 days they were necrotic; at what stage the necrosis developed was uncertain. They also failed to respond to hypervitaminosis A when incubated on AH medium, although controls from the limb removed before the embryo was heated gave the characteristic reaction (Pl. 3, cf. figs. 23 and 24, and 25 and 26). On the other hand, preheating to 40° C. prevented neither growth in normal medium nor the typical response to hypervitaminosis A (Pl. 3, cf. figs. 27 and 28).

It is concluded that preheating the bones to a temperature that is just sufficient to inhibit growth, also inhibits the vitamin A reaction.

The effect of heating the plasma of the culture medium. Blood plasma was heated to 50° C. for 15 min. in a water-bath. To part of the plasma, about 2280 i.u. vitamin A/100 ml. was added before heating.

Heating the plasma affected neither the growth of the bones nor their response to hypervitaminosis A. From this it is concluded that nothing essential for either the growth of the bones or their reaction to hypervitaminosis A was destroyed.

IV. *A comparison of the effects on explanted foetal mouse bones of 'natural' and 'artificial' hypervitaminosis A*

In the experiments described in the preceding sections, the hypervitaminosis was 'artificially' induced by adding vitamin A acetate or alcohol to normal plasma. The following experiments were made to see whether the effect on explanted mouse bones of vitamin A, added to normal plasma in this way, was the same as that of a similar concentration of vitamin A introduced 'naturally' into the plasma by feeding the donor fowl on a diet rich in the vitamin.

Experiments were also undertaken to see whether there was any difference in the state of the vitamin in the plasma in 'artificial' and in 'natural' hypervitaminosis respectively.

The effect of 'natural' and 'artificial' hypervitaminosis A on late foetal mouse bones. Bone rudiments from mouse embryos near term, similar to those described in section II, were used. Three experiments were made (Exps. 16, 19 and 23, Table 3), in which the effects on the explants of plasma from a fowl having hypervitaminosis A (NH medium) was compared with that of normal plasma to which approximately the same quantity of vitamin A acetate or alcohol had been added (AH medium).

Since the 'artificial' hypervitaminosis had been produced in Exps. 16 and 19 by adding a solution of crystalline vitamin A acetate to the medium and in Exp. 23 by adding vitamin A alcohol extracted from avoleum (see p. 322), a fourth (control) experiment was made in which the effects of these two substances on explanted mouse bones were directly compared. Ten pairs of foetal mouse bones were grown as follows. Four pairs: one of each pair was cultivated in medium containing about 1500 i.u. of vitamin A acetate per 100 ml. and the other in normal medium; four pairs: one of each pair was grown in medium containing about 1500 i.u. of vitamin A alcohol from avoleum per 100 ml. plasma, and the other in normal medium; two pairs: one of each pair was grown in medium to which vitamin A acetate had been added and the other in medium containing vitamin A alcohol.

The results of the first three experiments are summarized in Table 3. In the first experiment (Exp. 16), even after 12 days' cultivation in the NH medium, the bones seemed unaffected when compared with their controls grown in normal medium. The only exceptions were the two fibulae in which there was more absorption and less deposition of bone than in the corresponding controls.

The 'natural' hypervitaminosis produced some effect in the second experiment (Exp. 19) but far less than did the equivalent 'artificial' hypervitaminosis. Apart from a slight rarefaction of the bone, the explants in NH medium showed little change during the first 7 days, but by the 12th day they had shrunk to about two-thirds the size of their normal controls, the bone was much eroded while the cartilaginous ends had dwindled and the matrix had

TABLE 3. Comparison of the effects of 'natural' and 'artificial' hypervitaminosis A on foetal mouse bones *in vitro*

(The foetuses used for Exp. 23 were younger than those used in Exps. 16 and 19. NH, plasma from a fowl with hypervitaminosis; AH, plasma with added vitamin A; N, normal plasma)

Exp.	Vitamin A (i.u./100 ml. plasma)	Days in culture	No. of explants	Effect of hypervitaminosis
16	NH: 1435	5	4: tibiae, fibulae, radius, ulna	No difference between NH and N except in fibula where shaft is rather disorganized in NH
	N: 487	5	4: tibiae, fibulae, radius, ulna	
	NH	12	4: tibiae, fibulae, radius, ulna	More absorption and less deposition of bone in fibula in NH than in N; otherwise no difference between NH and N
	N	12	4: tibiae, fibulae, radius, ulna	
19	NH: 1235, 1545	7	4: 2 radii, 2 ulnae	NH: little or no difference between NH and N
	AH: 1287, 1490 (vit. A acetate)	7	4: 2 radii, 2 ulnae	AH: very great reduction of cartilage; basophilia of matrix almost or completely lost; considerable absorption of bone
	N: 327, 296	7	4: 2 radii, 2 ulnae	
	NH	12	4: 2 tibiae, 2 fibulae	NH: marked difference from N; cartilage greatly reduced; partial loss of basophilia; much bone absorption
	AH	12	4: 2 tibiae, 2 fibulae	AH: Cartilage nearly (tibiae) or completely gone (fibulae); basophilia quite gone from matrix; bone nearly (tibiae) or completely gone (fibulae)
	N	12	4: 2 tibiae, 2 fibulae	
23	NH: 1540, 1800	4	4: 2 radii, 2 ulnae	NH: Cartilage slightly reduced as compared with N; slight loss of basophilia from matrix; much bone absorption
	AH: 1537, 1760 (vit. A alc.)	4	4: 2 radii, 2 ulnae	AH: Cartilage greatly reduced; partial loss of basophilia from matrix; extensive absorption and partial disruption of bone
	N: 200, 232	4	4: 2 radii, 2 ulnae	
	NH	7	4: 2 tibiae, 2 fibulae	NH: cartilage reduced; basophilia of matrix nearly or completely gone; bone partly absorbed
	AH	7	4: 2 tibiae, 2 fibulae	AH: much greater reduction of cartilage and absorption of bone than in NH
	N	7	4: 2 tibiae, 2 fibulae	N: slight rarefaction of bone, otherwise unchanged

begun to lose its basophilia (Pl. 3, cf. figs. 31 and 32). In similar bones grown for the same time in AH medium, the changes were much more advanced.

The greatest effect of 'natural' hypervitaminosis was obtained in the third experiment (Exp. 23), in which the concentration of vitamin A was slightly higher than in the two previous experiments (see Table 3) and the foetal bones were rather younger and therefore more susceptible to the action of the vitamin (see p. 335). There was, however, a great difference between the effects of the 'natural' and of the 'artificial' hypervitaminosis. By the 4th day, the bones had begun to shrink in response to both the 'natural' and the 'artificial' hypervitaminosis, but the shrinkage was far greater in the latter; similarly, while there was considerable bone absorption in the former, in the latter the shaft had largely disintegrated. The loss of basophilia from the cartilage matrix was always much more advanced in the 'artificial' than in the 'natural' hypervitaminosis.

In the fourth experiment in which the effects of vitamin A acetate and vitamin A alcohol were directly compared, the results showed little difference in the action of the two substances on explanted mouse bones, though if anything the vitamin A alcohol was slightly the more potent.

It is concluded that the effect of 'natural' hypervitaminosis A on explanted foetal mouse bones is similar to but much less drastic than an equivalent 'artificial' hypervitaminosis A.

The physical condition of vitamin A in the plasma in 'artificial' and 'natural' hypervitaminosis. Extraction experiments showed that by shaking the plasma with petrol ether, much of the vitamin A could be recovered from the AH plasma, whether it had been added as the acetate or the alcohol, but not from the NH plasma. On the other hand, if the NH plasma were first treated with 50% alcohol to denature the protein, the vitamin could then be extracted with petrol ether (Dzialoszynski, Mystkowski & Stewart, 1945). In the body it is probable that vitamin A is combined with a protein; if so, this combination may have to be broken down before the vitamin becomes soluble in petrol ether.

It may be concluded that the state of vitamin A added to plasma *in vitro* differs from that found in the fowl plasma as a result of hypervitaminosis A.

DISCUSSION

The first question to consider is how the effects of hypervitaminosis A on long-bone rudiments in culture compare with its effects on the long bones of young animals. Wolbach (1947) has recorded the following changes in the limb bones of young hypervitaminotic guinea-pigs and rats. For the cartilage: (1) rapid maturation of the cartilage cells, so that vesicular chondroblasts extend far towards the epiphysial line; (2) relative retardation of matrix-formation, causing compression of the maturing cells on the diaphysial side

of the cartilage plate; (3) cessation of cell division in the proliferative cartilage; (4) very deep and rapid penetration of the cartilage by capillaries; (5) concurrently rapid replacement of the cartilage by bone, resulting in premature closure of the epiphyses and arrest of linear growth. In the bone he observed: (1) acceleration of remodelling sequences, involving absorption by increased osteoclasis and appositional bone formation; absorption and deposition take place at the same sites as in normal bone development; (2) failure of the matrix of the newly formed bone to mature and to calcify completely; (3) fractures caused by excessive absorption of the old cortical bone and lack of firmness in the new bone. There is a quantitative relationship between the rate at which these changes appear in the long bones and the dose of vitamin A received by the animal.

Wolbach has shown that the changes produced in the limb bones *in vivo* by hypervitaminosis A are closely related to the normal growth pattern of the bones. Hence, when comparing the effects of excess vitamin A on the explanted bone rudiments *in vitro* and on the long bones of young animals, certain important differences between the growth patterns of the bones *in vitro* and *in vivo* must be borne in mind.

Limb-bone rudiments of 5- to 6-day chick embryos grown *in vitro* do not acquire a marrow cavity by excavation of the cartilage nor is the bone subjected to remodelling sequences by absorption and redeposition as in normal development *in ovo*. On the other hand, the young cartilage differentiates normally into hypertrophic region, zones of flattened cells and epiphyses, and a fairly thick single layer of bone is deposited on the surface. When hypervitaminosis A is imposed on this developmental picture, the result resembles somewhat that obtained *in vivo* in that linear growth is arrested, spontaneous fractures occur, differentiation of the chondroblasts is not retarded, though there is no conclusive evidence that it is accelerated, and ossification proceeds at almost the normal rate. The excavation of cartilage and the absorption of bone, processes which are omitted from the normal pattern of growth in these explants, are not induced by hypervitaminosis A, at least not during the culture periods of the present experiments. The most profound effect of the vitamin is on the cartilage matrix of chick explants. Whereas the spontaneous fractures which occur *in vivo* are due to the erosion of the old bone and the softness of the new, in the explants they are caused by softening of the intercellular material of the cartilage at its weakest point, i.e. just beyond the sheath of periosteal bone, so that it cannot withstand the pressure of the surrounding capsule of connective tissue. It is not clear whether the epiphysial cartilage is affected *in vivo* in any comparable way. Wolbach (1947) states that the formation of cartilage matrix in hypervitaminotic animals 'does not keep pace' with the rapid maturation of the cartilage cells 'and in consequence, there is compression of the maturing cells on the diaphysial side of the

cartilage plate'. This observation suggests that *in vivo* also, there may have been a slight softening and even reduction of the cartilage matrix, as well as retardation or inhibition of its production.

Late foetal mouse bones in culture present a different developmental pattern from that of the early chick rudiments. They are at an advanced stage of differentiation when explanted, with a marrow cavity, a stout shaft of bone and large cartilaginous ends. During cultivation they enlarge a little, but much less than do the chick rudiments. Their formative processes are greatly reduced *in vitro*, probably owing to the sudden abolition of a blood supply from such very vascular structures; endochondral ossification ceases and not much periosteal bone is formed. Excavation of the cartilage and absorption of bone continue, however, and most of the skeletal cells survive in a healthy condition, though in a state of depressed functional activity.

Hypervitaminosis A affects this histological picture rather differently from that of the chick explants. The absorption of cartilage and bone is enormously accelerated as *in vivo*, but the old bone is not replaced by new osteoid tissue as it is in the limb bones of the animal, so that in culture the shaft disappears altogether. The cartilage shows the same remarkable changes as appear in the hypervitaminotic chick rudiments, but in the mouse the dissolution of the matrix is so rapid and complete that in 7–10 days the intercellular material has gone entirely, leaving the chondroblasts naked.

It remains to consider why the vitamin should have a far more drastic effect on the explanted bone rudiments in our experiments than it has on the long bones of hypervitaminotic animals. There appear to be two main reasons, though other factors may also be concerned. In the first place, as shown in section IV, excess vitamin A introduced 'naturally' into the plasma by feeding the donor fowl has a much lower biological activity than when it is added to normal plasma, though its action on the explants is qualitatively similar in both cases. In the second place, the experiments in section II demonstrated that the bones of younger mouse embryos are much more susceptible to hypervitaminosis A than those of older foetuses, which in turn react more quickly than the bones of 3-day infant mice. It seems likely, therefore, that the bones of young weanling animals such as are used to study the action of hypervitaminosis A *in vivo*, would be still less sensitive to the vitamin.

The question arises as to why the bones of the younger foetuses should be more susceptible to hypervitaminosis A than those of older embryos and infant animals. It has been shown that cartilage killed by mechanical damage or by heating to a temperature (45° C.) just sufficient to kill the cells, fails to respond to vitamin A. From this we conclude that the effect of vitamin A is not directly on the intercellular material but depends on the activities of living cells. The younger the bone, the greater is the proportion of cellular to intercellular material, and the less are the hardness and density of the latter;

it is probably these factors which determine the degree of the effect of hypervitaminosis A.

Many aspects of this problem remain obscure. At present we do not know how the metabolism of the cells is altered by hypervitaminosis A, what chemical changes are produced in the cartilage matrix or how far the effects of the vitamin are reversible. Further studies are planned which we hope may shed light on these and other questions.

SUMMARY

1. Experiments were made to study the effect of hypervitaminosis A on embryonic long bones growing under the simplified environmental conditions provided by tissue culture.

2. Pairs of limb-bone rudiments from 5- to 6-day chick embryos and foetal mice near term, were cultivated by the watch-glass method. One of each pair was grown in hypervitaminotic and the other in normal medium.

3. In normal medium the chick rudiments sometimes grew to more than four times their original length, the cartilage differentiated into hypertrophic region, zones of flattened cells and epiphyses; a layer of periosteal bone was deposited but no marrow cavity was formed by excavation of the cartilage, as occurs *in vivo*.

4. In medium to which excess vitamin A alcohol had been added growth was arrested, but the cartilage differentiated at the normal rate and ossification was only slightly reduced. The cartilage matrix was greatly affected; it shrank, softened and lost its basophilia and metachromasia while its affinity for van Gieson's stain was much increased. There was no excavation of cartilage or absorption of bone. The surrounding soft tissue grew profusely.

5. These changes were not associated with cell degeneration.

6. The severity of the effect was correlated with the concentration of vitamin in the culture medium.

7. Late foetal mouse bones in normal medium enlarged, but much less than the chick rudiments; endochrondral ossification ceased, but a little more periosteal bone was formed in some explants. Excavation of the cartilage and absorption of the spongy bone continued *in vitro*; necrosis was usually slight.

8. The addition of vitamin A acetate or alcohol to the medium had a drastic effect on the mouse explants. The cartilage matrix rapidly dwindled, lost its metachromasia and finally disappeared, leaving the chondroblasts naked. The bone was quickly absorbed, though in most experiments few osteoclasts were present, it was not replaced by new osteoid tissue.

9. The younger the foetuses from which the long bones were obtained, the greater was the effect of the vitamin A.

10. The higher the concentration of added vitamin A in the medium, the more rapid was its effect on the explanted mouse bones.

11. Mechanically damaged cartilage, in which the cells were dead, did not respond to hypervitaminosis A like the adjacent living cartilage.

12. Heating the mouse bones to 45° C. prevented their growth in normal medium and their characteristic reaction to hypervitaminosis A; heating to 40° C. did not affect either their growth in normal medium or their response to hypervitaminosis A.

13. 'Natural' hypervitaminosis A obtained by using plasma from a hypervitaminotic fowl for the culture medium, had a qualitatively similar but far less drastic effect on explanted foetal mouse bones than 'artificial' hypervitaminosis A produced by adding the same quantity of vitamin A acetate or alcohol to normal plasma.

14. Extraction experiments showed that whereas much of the added vitamin A can be removed from the plasma by petrol ether, it cannot be extracted from the 'natural' hypervitaminotic plasma unless the protein has first been denatured with 50% alcohol.

15. It was concluded that: (*a*) vitamin A in concentrations similar to those produced in the blood of hypervitaminotic animals, has a direct action on cartilage and bone *in vitro*; (*b*) the severity of the effect depends on (1) the concentration of the vitamin in the culture medium and (2) the proportion of skeletal cells to intercellular material in the explanted rudiment; (*c*) the character of the vitamin A effect is correlated with the developmental pattern of the explant under normal conditions of culture, and is probably an exaggerated form of that produced in the bones of young animals by hypervitaminosis A; (*d*) vitamin A has a lower biological activity and is in a different state in the blood plasma of a hypervitaminotic animal than when it is added directly to normal plasma.

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EXPLANATION OF PLATES

The photomicrographs were taken by Mr V. C. Norfield (Strangeways Research Laboratory), except figs. 23–28 which were the work of Mr R. J. C. Stewart (National Institute for Medical Research).

Abbreviations

dc	degenerate cells	*pb*	periosteal bone
ep	epiphysis	*pf*	pressure fibrils
m	metachromasia	*zfc*	zone of flattened cells
mdc	mechanically damaged cartilage	*zhc*	zone of hypertrophic cells

Plate 1

Fig. 1. Femur from a 6-day chick embryo at stage 3 (see p. 324). Hypertrophy of the cartilage cells is just beginning in the middle segment of the shaft, but no bone has formed. ×19. (Delafield's haematoxylin, chromotrop.)

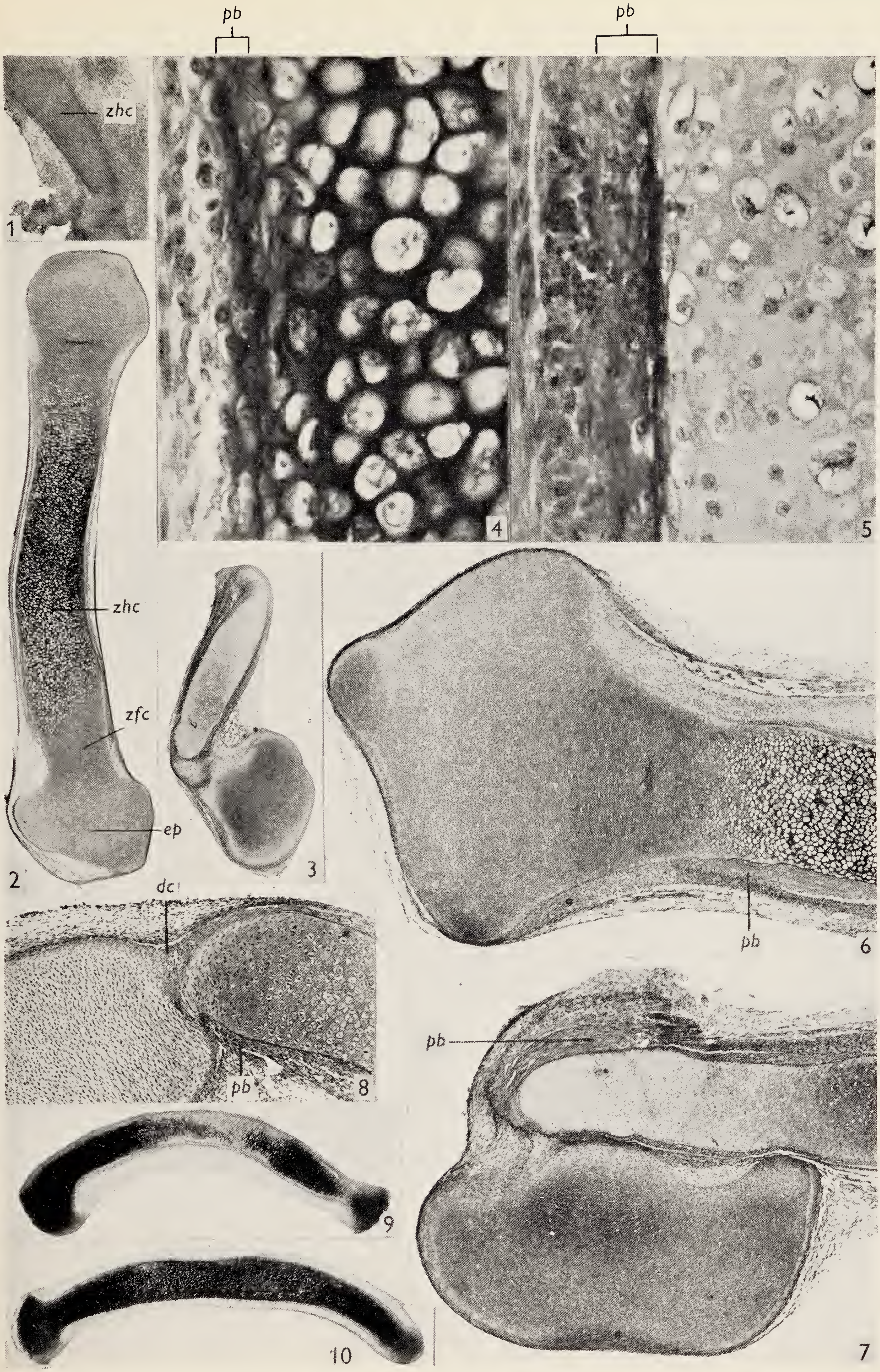
Fig. 2. Control femur (Exp. 46) explanted at a slightly earlier stage (stage 2) than that shown in fig. 1, and cultivated in normal medium for 9 days. (Camera lucida drawings of this femur in life are shown in Text-fig. 1). Note the great enlargement that has taken place *in vitro*, and the differentiation of the cartilage into hypertrophic region, zones of flattened cells and epiphyses. The cartilage matrix is strongly basophilic. ×19. (Delafield's haematoxylin, chromotrop.)

Fig. 3. Femur from the opposite side of the same chick as shown in Fig. 2, after 9 days' cultivation in AH medium (see Text-fig. 1). Note the small size of the explant and the loss of basophilia from the matrix; the condylar end is nearly detached from the shaft. ×19. (Delafield's haematoxylin, chromotrop.)

Fig. 4. Part of the diaphysial region of the section shown in fig. 2. Note the layer of periosteal bone which has been deposited on the surface of the cartilage, the vacuolated hypertrophic chondroblasts and the broad intercellular partitions of strongly basophilic matrix. ×400.

Fig. 5. Part of the diaphysial region of the section shown in fig. 3. Note the periosteal bone and the loss of basophilia from the cartilage matrix; the chondroblasts are smaller and less vacuolated than those of fig. 4, but have not degenerated. ×400.

Fig. 6. Proximal end of a control humerus (Exp. 46) from a chick embryo at stage 2, after 11 days' cultivation in normal medium (see Text-fig. 2). The three zones of cartilage are highly developed and a fairly thick layer of periosteal bone has been formed. ×37. (Delafield's haematoxylin, chromotrop.)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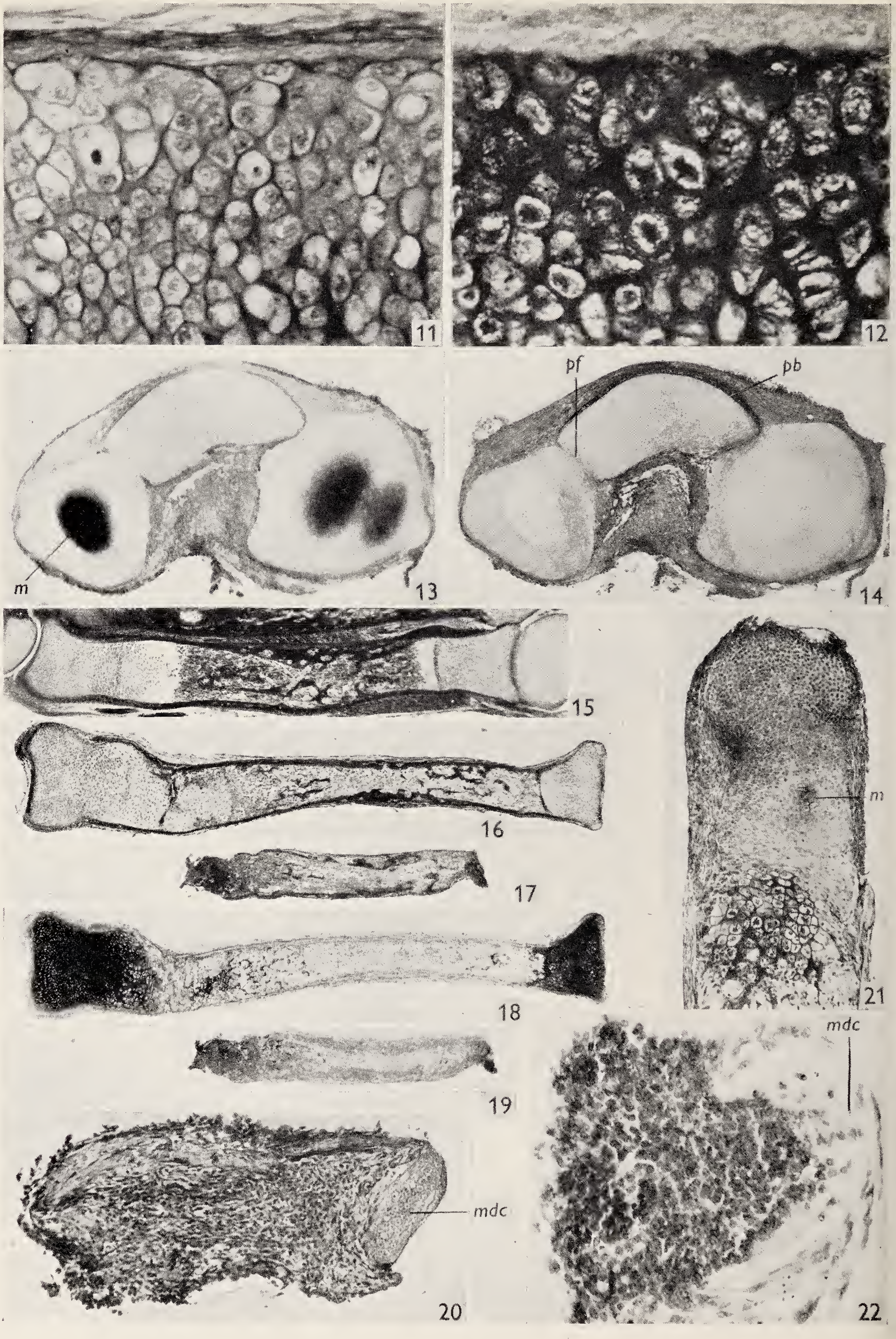
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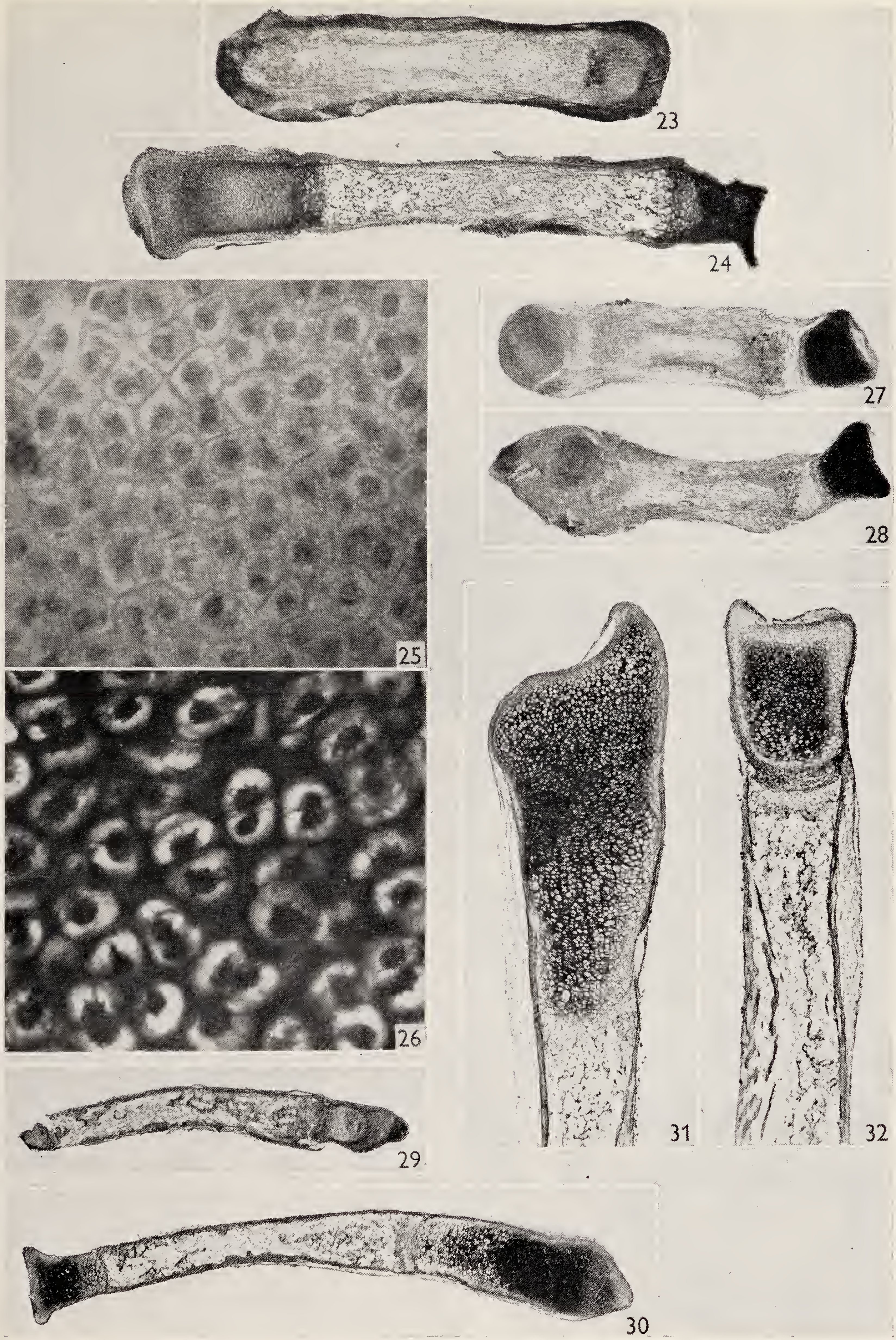
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Fig. 7. Proximal end of the opposite humerus from the same embryo as that shown in fig. 6, after 11 days' cultivation in AH medium (see Text-fig. 2). The end of the humerus has become detached and displaced, the periosteal bone is spreading diffusely from the broken end of the shaft and the cartilage matrix of the hypertrophic region has lost much of its basophilia. ×37. (Delafield's haematoxylin, chromotrop.)

Fig. 8. Proximal end of a humerus (Exp. 43) from a chick embryo at stage 3, after 4 days in AH medium. This shows an early stage in the detachment of the end; the shaft is constricted just beyond the proximal limit of the periosteal bone, the cartilage cells are compressed and some are degenerate. ×52. (Delafield's haematoxylin, chromotrop.)

Fig. 9. Radius (Exp. 47) from a chick embryo at stage 4, after 7 days' cultivation in AH medium. Metachromasia has begun to disappear from the peripheral cartilage of the shaft. ×19. (Toluidine blue.)

Fig. 10. Control radius from the same embryo as that shown in fig. 9, after 7 days' cultivation in normal medium. Note the intense metachromasia of the cartilage matrix. ×19. (Toluidine blue.)

Plate 2

Fig. 11. Part of the diaphysial region of the section shown in fig. 9. Note the narrow intercellular partitions in the cartilage, and the compact arrangement and healthy appearance of the chondroblasts. ×400.

Fig. 12. Part of the diaphysial region of the section shown in fig. 10. Note the broad, intensely staining metachromatic intercellular partitions; the shape of the chondroblasts is less regular than in the hypervitaminotic cartilage shown in fig. 11. ×400.

Fig. 13. Humerus (Exp. 50) from a chick embryo at stage 2, after 11 days' cultivation in AH medium. One end is completely and the other nearly detached and metachromasia has disappeared from the cartilage except in the interior of the two ends. Most of the cells in this explant appeared healthy. ×24. (Toluidine blue.)

Fig. 14. Section of the same humerus as that shown in fig. 13, stained with celestine blue, haematin and van Gieson's solution. All the cartilage stains bright pink with van Gieson's stain except the two regions which in the previous section retained their metachromasia; note the pressure fibrils in the cartilage at either end of the shaft. ×24.

Fig. 15. Normal radius (Exp. 52) from a late mouse foetus. Note the large terminal cartilages, stout shaft of bone and the marrow cavity permeated by endochondral bone trabeculae. ×23. (Celestine blue, haematin, van Gieson.)

Fig. 16. Control radius (Exp. 52) from a late mouse foetus after 7 days' cultivation in normal medium (see Text-fig. 3). The rudiment has enlarged slightly, much of the endochondral bone has been absorbed and endochondral ossification has ceased; the terminal cartilage is being invaded in an irregular way by tissue from the marrow cavity. ×23. (Celestine blue, haematin, van Gieson.)

Fig. 17. Opposite radius from the same foetus as the control shown in Fig. 16, after 7 days' cultivation in AH medium (see Text-fig. 3). Note the enormous shrinkage of the entire rudiment, the extensive absorption of the bone and the density of the marrow reticulum. ×23. (Celestine blue, haematin, van Gieson.)

Fig. 18. The same control as that seen in fig. 16, showing the large, metachromatic terminal cartilages. ×23. (Toluidine blue.)

Fig. 19. The same hypervitaminotic radius as that shown in fig. 17. The terminal cartilage has disappeared. ×23. (Toluidine blue.)

Fig. 20. Radius of foetal mouse (Exp. 7) after 10 days' cultivation in AH medium. Nothing of the original rudiment remains except a nodule of mechanically damaged cartilage and a few spicules of bone. ×82. (Delafield's haematoxylin, chromotrop.)

Fig. 21. Terminal cartilage of foetal mouse radius (Exp. 52) after 4 days' cultivation in AH medium. The cartilage matrix has almost completely lost its metachromasia which persists only in a small internal area and in the remains of the hypertrophic zone. ×72. (Toluidine blue.)

Fig. 22. Terminal cartilage of foetal mouse radius (Exp. 51) after 4 days' cultivation in AH medium. Except in a crescent of mechanically damaged cartilage on the right, the matrix has completely disappeared and the liberated chondroblasts, which appear healthy, seem to be wandering away from the surface of the explant. ×220. (Toluidine blue.)

Plate 3

Fig. 23. Unheated radius of foetal mouse cultivated in AH medium for 4 days. The cartilage has nearly gone and the bone is much eroded. ×34. (Thionine, eosin.)

Fig. 24. Radius from the opposite side of the same mouse, heated at 45° C. for 15 min. and then cultivated for 4 days in AH medium. This explant did not grow and failed to respond to the hypervitaminosis A. ×34. (Thionine, eosin.)

Fig. 25. An area of cartilage in the section shown in fig. 23. The matrix has almost disappeared, but the chondroblasts look healthy. ×565.

Fig. 26. An area of cartilage in the section shown in fig. 24. There is no loss of matrix, but the chondroblasts are necrotic. ×565.

Fig. 27. Unheated radius of foetal mouse cultivated in AH medium for 4 days. The rudiment shows the characteristic response to hypervitaminosis A. ×34. (Thionine, eosin.)

Fig. 28. Radius from the opposite side of the same foetus, heated at 40° C. for 15 min. and cultivated in AH medium for 4 days. Heating at 40° C. has not affected the response of the explant to hypervitaminosis A. ×34. (Thionine, eosin.)

Fig. 29. Radius from a mouse foetus (Exp. 19) after 7 days' cultivation in 'artificial' hypervitaminosis. The explant shows the usual response to excess vitamin A added to the medium. ×23. (Delafield's haematoxylin, chromotrop.)

Fig. 30. Radius from the same foetus after 7 days' cultivation in 'natural' hypervitaminosis. Although the medium contained approximately the same quantity of vitamin A as was added to the AH medium, the rudiment appears unaffected. ×23. (Delafield's haematoxylin, chromotrop.)

Fig. 31. Distal end of a control tibia of a mouse foetus from the same experiment (Exp. 19) as the explants shown in the two preceding figures. The explant was grown in normal medium for 12 days. Note the large terminal cartilage with strongly basophilic matrix. ×35. (Delafield's haematoxylin, chromotrop.)

Fig. 32. Distal end of the opposite tibia of the same foetus as that shown in fig. 31, after 12 days' cultivation in NH medium. Note the greatly reduced cartilage, the matrix of which is beginning to lose its basophilia near the margin. ×35. (Delafield's haematoxylin, chromotrop.)